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14. ABSTRACT
This project focused on the novel finding from our group that the serine-threonine kinase Akt1 partitions into specialized membrane microdomains, termed lipid rafts, and that this localization event strongly influences the nature of Akt1 signaling. Lipid rafts are cholesterol-enriched membrane microdomains that serve as signal transduction platforms by sequestering and excluding signaling proteins and by harboring multi-protein complexes. Evidence was presented in the original proposal that in prostate cancer cells critical cell survival cues are processed via lipid rafts, which are dependent on cholesterol for signal transduction. This is a significant finding because the Akt1 kinase is a central signaling protein that is frequently activated in prostate cancer. I have hypothesized in this project that cholesterol accumulation in prostate cancer cells may promote oncogenesis by altering the nature of Akt1 signals that flow through lipid raft microdomains. The purpose of this project was to identify the mechanism of Akt1 recruitment to cholesterol-rich microdomains and to explore the biological consequences for regulation of this important kinase. Several new lines of evidence consistent with my hypothesis have been produced in the second year (including the two four-month EWFs) and are described and summarized in this revised final addendum report.

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"Membrane Heterogeneity in Akt Activation in Prostate Cancer"
Grant number: W81XWH-07-1-0148
Revised Final Addendum Report for 02/15/2007 - 10/14/2009 (incl. 2 four-month EWFs)

INTRODUCTION

This project focused on the novel finding from our group that the serine-threonine kinase Akt1 partitions into specialized membrane microdomains, termed lipid rafts, and that this localization event strongly influences the nature of Akt1 signaling. Lipid rafts are cholesterol- and sphingolipid-enriched membrane microdomains that serve as signal transduction platforms by sequestering and excluding signaling proteins and by harboring pre-formed multi-protein complexes. Evidence was presented in the original proposal that in prostate cancer cells critical cell survival cues are processed via lipid rafts, which are dependent on cholesterol for signal transduction. This is a significant finding because the Akt1 kinase is a central signaling protein that is frequently activated in prostate cancer. I have hypothesized in this project that cholesterol accumulation in prostate cancer cells may promote oncogenesis by altering the nature of Akt1 signals that flow through lipid raft microdomains. The purpose of this project was to identify the mechanism of Akt1 recruitment to cholesterol-rich microdomains and to explore the biological consequences for regulation of this important kinase.

BODY

Task 1 and 2: Determine the unique functional consequences of Akt recruitment to lipid rafts for signaling processes in prostate cancer cells.

Task 1 and Task 2 have been completed. I demonstrated the construction and expression of raft-targeted as well as raft-excluded Akt molecules (Fig. 1) These various constructs were then tested regarding their lipid raft distribution as proposed in Task 2 i).

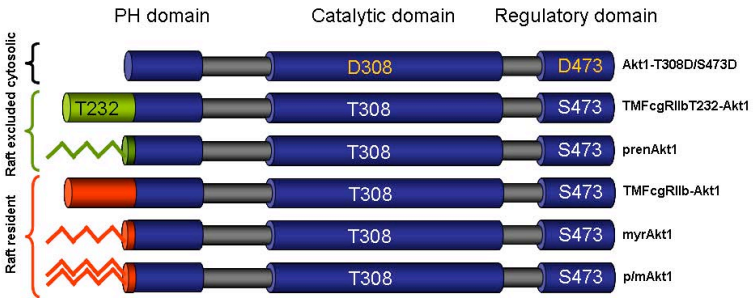

Figure 1: Overview of differentially targeted Akt1 fusion proteins. The Akt1 kinase features three characteristic domains including the pleckstrin homology (PH) domain, the catalytic domain with the Thr308 phosphorylation site and the regulatory domain with its Ser473 phosphorylation site. The PH domain of Akt1 is fused N-terminally to various targeting sequences that will guide Akt1 to rafts (red) or exclude it from this membrane compartment (green).

The lipid raft distribution of the Akt-T308D/S473D version (uppermost construct, Fig. 1), which represents activated Akt1 that resides in the cytoplasm, was tested. I included other Akt1 mutants in this study to see if a combination of various mutations would have any impact on the lipid raft distribution.

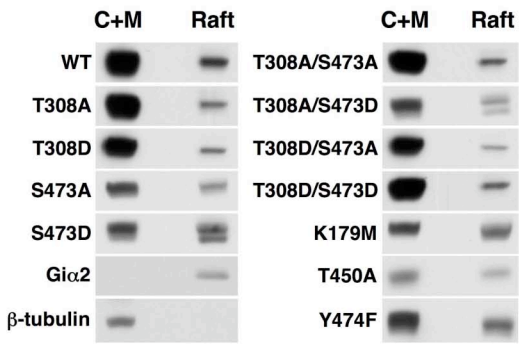

Figure 2: Lipid rafts contain a population of Akt1 kinases. Comparison of cytoplasmic/non-raft membrane fractions (C+M) and raft fractions after differential extraction reveals that partitioning of Akt1 into the lipid raft compartment is not influenced by mutating the 2 major regulatory sites of Akt1- threonine 308 and serine 473. Note that generation of a phosphomimetic at position 473 alone leads to increased accumulation of Akt1 in the lipid raft compartment. Interestingly, the kinase-dead mutant of Akt1 (K179M) is predominantly present in lipid rafts.

As seen in Fig. 2, all mutants including the constitutively active Akt-T308D/S473D were successfully expressed in HEK293 cells. The specific subcellular localization of each mutant was verified by differential extraction with Triton X-100 and octylglucoside detergents and revealed that all Akt1 mutants partitioned into lipid raft microdomains as demonstrated by the prevalence of G-protein subunit α-2 and the absence of the non-raft marker β-tubulin. Comparison of cytoplasmic/non-raft membrane fractions and raft-fractions after differential extraction revealed that partitioning of Akt1 into the lipid raft compartment is not influenced by mutating the two major regulatory sites threonine 308 and serine 473. The phosphomimetic double mutant Akt-T308D/S473D (uppermost construct, Fig. 1) did not show a lipid raft distribution that was different from the wild type and proved to be well suited as an activated control construct. Interestingly, the Akt-S473D phosphomimetic mutant (not included in the original proposal), displayed altered distribution with increased accumulation of the kinase in the lipid raft compartment. Moreover, the kinase-dead mutant of Akt1 (K179M), which is unable to bind ATP in the active site, accumulated predominantly in lipid rafts. Consequently, I was able to identify an important function for serine 473 and lysine 179 in regulating the admission of Akt1 to the lipid raft signaling platform.

Cloning and expression of the other five proposed Akt1 variants (see Fig. 1) was also completed (Task 1. ii) and iii)). Of these constructs, the most promising pair turned out to be a N-terminal fusion of the Akt1 PH-domain to the transmembrane domain of the FcγRIIb receptor (TMFcγRIIb-Akt1 and TMFcγRIIbT_{232}-Akt, 2nd and 4th construct from the top, Fig. 1). After raft fraction isolation using our established SDEM protocol to study subcellular localization (Task 2. i), I was able to demonstrate that TMFcγRIIb-Akt1 was clearly enriched in raft membranes (Fig. 3). By introduction of a single amino acid substitution into the transmembrane domain of TMFcγRIIb-Akt1 by site-directed mutagenesis (TMFcγRIIbT_{232}-Akt, 2nd from the top, Fig. 1), I further demonstrated virtually complete exclusion of this Akt1 version from the lipid raft compartment (Fig. 3, right).

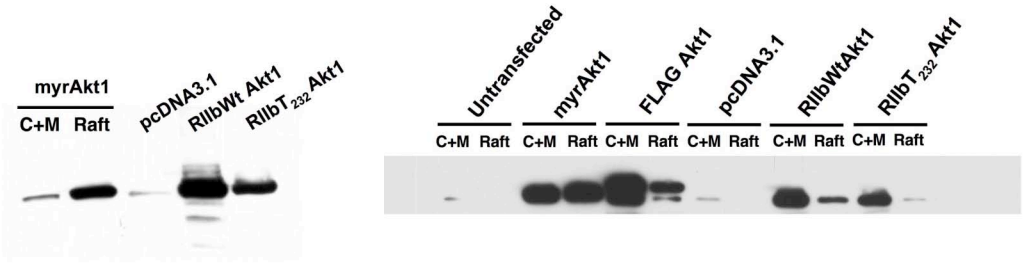

Figure 3: Generation of raft-resident and raft-excluded variant of Akt1. Left: Expression of a raft-targeted version of Akt1 (RIIbWtAkt1) and a raft-excluded version of Akt1 ($RIIbT_{232}$Akt1) in HEK293 cells. Right: Verification of subcellular localization by differential extraction shows that the myristoylated form of Akt1 is highly enriched in lipid rafts and that FLAG-tagged wild-type Akt1 partitions into the raft compartment. The transmembrane domain of the FcγRIIb receptor is able to target Akt1 to lipid rafts and a single point mutation at position 232 abolishes this effect.

Unfortunately, the proposed alternative approach to create a raft-excluded Akt1 variant (third construct from the top, Fig. 1), proved to be incompatible with Akt1 activity. The prenylation signal derived from K-Ras rendered Akt1 catalytically inactive when fused to the N-terminal PH-domain (Fig. 1). Fusion of this motif to the Akt C-terminus resulted in very low expression levels (Fig. 4).
Despite this setback, I was able to generate a complete set of constructs to study the consequences of raft-targeting and raft-exclusion of the Akt1 kinase for cancer cell proliferation and cell survival.

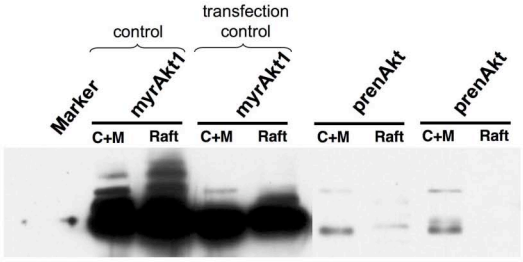

Figure 4: Differential extraction of a raft-excluded version of Akt1. Addition of a prenylation sequence to Akt1, which is predicted to exclude Akt1 from rafts results in very low expression levels in comparison to the raft-targeted Akt1 version.

I have also answered the question how signals transmitted from raft-resident Akt affect discrete cellular behaviors (Task 2. ii)). First, I focused on the sensitivity of raft-resident Akt1 to lipid raft disruption using the cholesterol binding drug methyl-β-cyclodextrin (CD). To confirm the cholesterol sensitivity of lipid raft-resident Akt1, LNCaP cells were treated with CD and fractionated into cytosol/ nonraft membranes (C+M) and raft membranes. Akt1 was immunoprecipitated from each fraction and blotted with antibodies to total and phospho-S473 Akt1. Cyclodextrin treatment did not appreciably alter the amount or extent of phosphorylation of Akt isolated from the C+M fraction. In contrast, CD ablated phosphorylation of raft-resident Akt1 (Fig. 5).

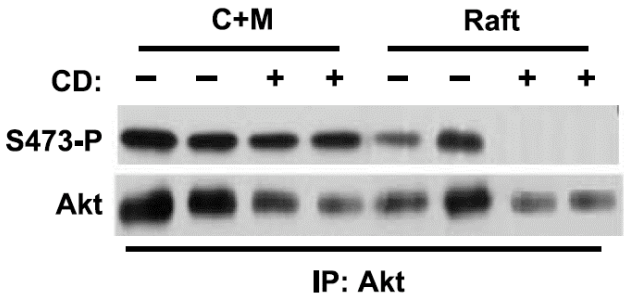

Figure 5: A population of endogenous Akt1 resides in a cholesterol-rich membrane fraction. Akt1 was immunoprecipitated from LNCaP cells exposed to cyclodextrin (CD) and fractionated into C+M and raft fractions. Immunoprecipitated eluates were blotted with antibodies to total and phospho-Akt1.

After having established that the raft-resident population is especially sensitive to lipid raft disruption using CD, I further elucidated whether this sensitivity is reflected in the susceptibility to apoptotic triggers such as the PI3-kinase inhibitor LY294002, which I had proposed in Task 2 ii). This reflection was based on a previous finding from our group that demonstrated the induction of apoptosis in LNCaP cells after treatment with LY294002. Therefore, stably transfected LNCaP prostate cancer cells were generated, which express a myristoylated form of Akt1 that significantly enriches Akt1 in rafts (myrAkt, construct #5 from top, Fig. 1). Sequences encoding LacZ (control) or myrAkt1 were cloned into pLenti6/V5-DEST and transfected into HEK293T cells for virus production. Lentiviruses were collected and used for infection of LNCaP prostate cancer cells. After selection of blasticidin-resistant cells, stable transformed clones were isolated and further analyzed. With these stably transfected cell lines I developed and optimized an assay to determine the biological consequences of membrane heterogeneity of Akt1 activation.

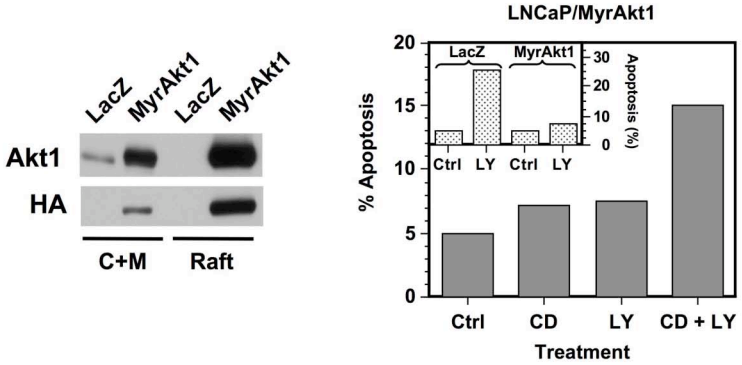

Figure 6: Activated Akt1 confers resistance to apoptosis induced by PI3K inhibition. Stable expression of myristoylated Akt1 in lentivirus-transfected LNCaP cells shows considerable enrichment of Akt1 in lipid rafts in comparison to the control (LacZ). LNCaP cells stably expressing myrAkt1 or control cells expressing LacZ (inset) were treated without or with LY294002 for 24 hours and the extent of apoptosis determined by flow cytometry. LNCaP/myrAkt1 cells were treated without (Ctrl) or with 5mM cyclodextrin (CD) for 1 hour, 10uM LY294002 (LY) for 24 hours or both agents (CD + LY) and harvested for flow cytometry. Data are presented as apoptotic cells (sub-G1-peak) expressed as a percentage of the total cell population and are representative of two independent trials.

As shown in Figure 6, enrichment of myrAkt1 within lipid rafts was confirmed in the isolated clones, which were then chosen to further elucidate whether raft-resident Akt1 could function to promote cell survival. Stably transformed LNCaP/MyrAkt1 and control cells expressing LacZ were exposed to the PI3K inhibitor LY294002 for 24hours and its

apoptotic effect on LNCaP cells was assessed by flow cytometry (Fig. 6). Surprisingly, LNCaP/MyrAkt cells were almost completely insensitive to PI3K inhibition, in contrast to LNCaP/LacZ cells that displayed significant induction of apoptosis (inset). However, the cytoprotective effect of MyrAkt1 was diminished by depletion of membrane cholesterol prior to treatment with LY294002, suggesting that anti-apoptotic signals are transmitted by the raft-resident population of Akt1.

To further understand how signals transmitted from raft-resident Akt1 differ from Akt signaling at other membrane locations, I used the stably transfected LNCaP prostate cancer cells expressing a raft-targeted form of Akt1 (see above). The LNCaP prostate cancer cells expressing this raft-resident MyrAkt1 (second construct from the bottom, Fig. 1) were tested regarding their cholesterol sensitivity by manipulating membrane cholesterol levels with either cyclodextrin alone, water-soluble cholesterol alone or cyclodextrin treatment followed by restoring membrane cholesterol. As shown in Figure 7, CD-treatment led to a decrease in membrane cholesterol compared to untreated cells and essentially a complete loss of MyrAkt1 from rafts.

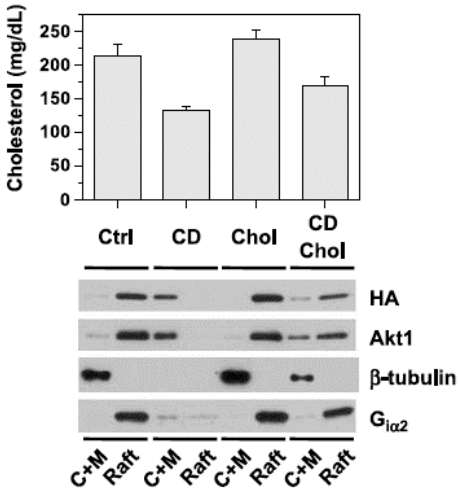

Figure 7: Oncogenic Akt1 is enriched in lipid rafts and sensitive to cyclodextrin. Stably transfected LNCaP cells expressing MyrAkt1 were treated with either cyclodextrin (CD) or water-soluble cholesterol (Chol) or with CD followed by cholesterol treatment (CD Chol). Cells incubated in serum-free medium served as controls. Following differential extraction of Triton-soluble cytoplasmic/non-raft membrane fractions and octylglucoside-soluble lipid raft fractions, proteins were blotted and detected with antibodies to Akt1, the HA-tag, tubulin and G-protein subunit $\alpha 2$ as the raft marker.

Cholesterol repletion restored membrane cholesterol levels to ~80% of the basal level and reestablished the basal distribution of MyrAkt1. Cholesterol treatment in the absence of depletion increased membrane cholesterol by ~12% and led to a modest but detectable increase of MyrAkt1 in rafts.

In a second set of experiments, the raft-resident population of Akt1 was further examined in regards to its sensitivity to specific inhibitory proteins, as proposed in Task 2 iii). Based on my hypothesis that Akt1 interacts with a specific set of regulatory proteins that are admitted to the lipid raft compartment, I decided to focus explicitly on inhibitory proteins that also show enrichment in rafts. Interestingly, our laboratory has begun to specifically explore the lipid raft microdomain in search for Akt1 interacting proteins that transit raft membranes. Immunoprecipitation of Akt1 from lipid raft fractions of LNCaP cells and subsequent analysis of co-precipitating proteins by MALDI-TOF mass spectrometry identified the mammalian serine/threonine sterile 20 (STE20)-like kinase Mst1 as a potential Akt1 interacting protein. Mst1 was detected primarily in Akt1 complexes isolated from raft fractions, which suggests that complex formation between endogenous Mst1 and Akt1, as measured by co-IP, occurs preferentially in lipid raft membrane frac-

tions (Fig. 8, left). Moreover, our laboratory recently reported that expression of Mst1 inhibits endogenous Akt1 activity in LNCaP cells and induces apoptosis, which is in agreement with the requirement for constitutive signaling through the PI3K/Akt pathway for survival in this cell line. Consequently, I decided to test the sensitivity of raft-resident Akt1 to the inhibitory activity of Mst1. As expected, PI3K inhibiton by the LY compound induced apoptosis in LNCaP cells (Fig. 8, right). Surprisingly, silencing of Mst1 expression by siRNA antagonized the apoptotic effect of LY, implicating Mst1 in the apoptotic mechanism induced by PI3K inhibition.

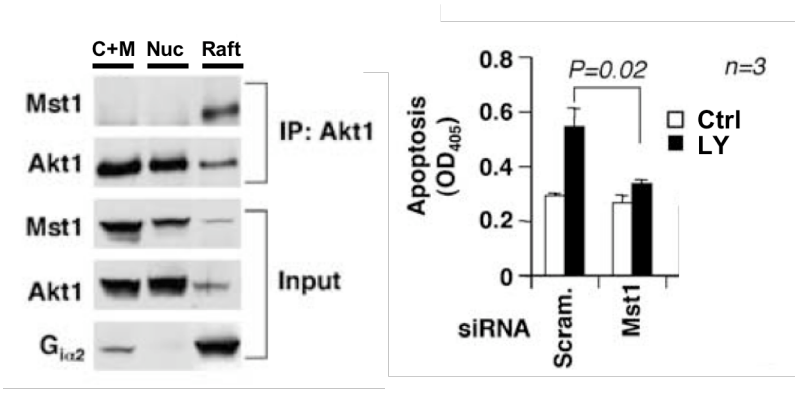

Figure 8: Mst1 and Akt1 interact preferentially in lipid raft fractions. Immunoprecipitated Akt1 from cytosol/non-raft membranes, nuclear or raft fractions displays complex formation with Mst1 only in the lipid raft domain (left). Induction of apopotosis in LNCaP cells by treatment with LY involves Mst1 and is diminished in absence of this kinase.

Task 3 and 4. Determine the functional role of HSP90 family members in Akt1 activity and recruitment to lipid rafts in prostate cancer cells.

To elucidate the underlying molecular mechanism of Akt1 recruitment to rafts, I generated FLAG epitope tagged versions of HSP90 and HSP89αΔN as proposed in Task 3. i). Total RNA from LNCaP, PC-3 and DU145 prostate cancer cells was isolated and RT-PCR with gene specific primers was subsequently performed to isolate the HSP89αΔN coding sequence (Fig. 9A). A PCR product in the expected size range was generated in LNCaP cells (band A) and DU145 (band B, C) cells but was not detected in PC-3 cells (Fig. 9B). TA-cloning and sequencing of the amplified fragment revealed that band A from LNCaP cells and band B from DU145 cells both mapped to a different isoform of HSP90 on chromosome 3 instead of chromosome 14. Band C turned out to be a non-specific amplification artifact (Fig. 9C).

Due to the difficulties I encountered with retrieving the cDNA of HSP89αΔN, I decided to clone this gene with a nested primer strategy. To this end, I designed two overlapping 5' PCR primers (Fig. 9, P1/P2) that together encode the entire N-terminal domain of HSP89αΔN and prime with the conserved part of the HSP90 gene that is identical to HSP89αΔN. Together with the HSP90 gene-specific 3' primer P3, the inner 5' primer (P1) was used in a RT-PCR reaction to amplify the HSP90 gene from total cDNA. Subsequently, the PCR product from this reaction served as a template to amplify the complete HSP89αΔN gene in a PCR reaction using primer pair P2/P3. As shown in Figure 9E, control restriction digest with BamHI/HindIII demonstrated a fragment of correct molecular weight that was confirmed by sequencing. Even though I was able to accomplish construction and cloning of the HSP89αΔN gene, I was not able to achieve expression of this construct in prostate cancer cells.

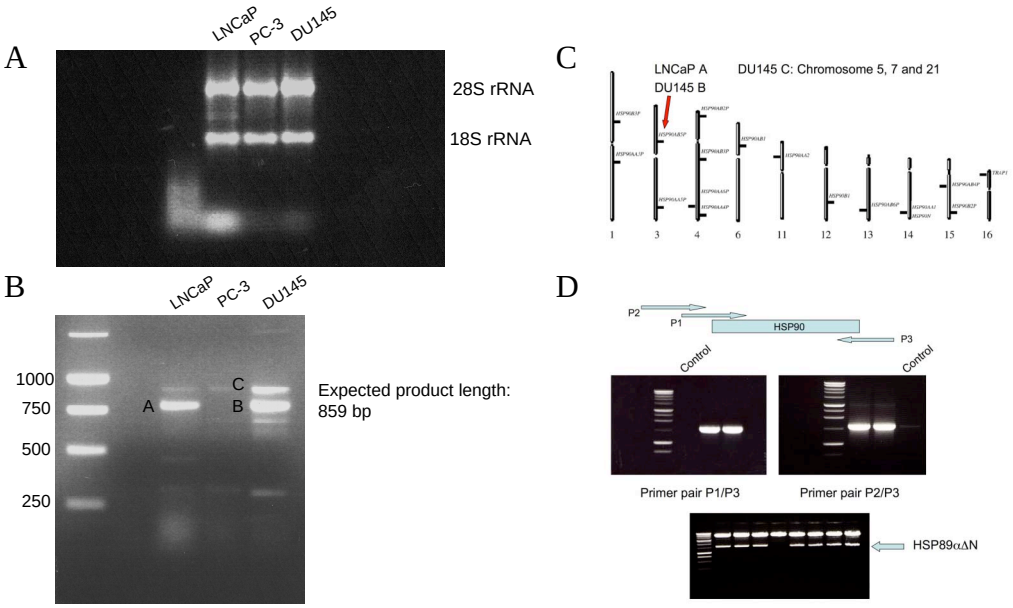

Figure 9: Cloning of HSP89αΔN. (A) Isolation of total RNA from LNCaP, PC-3 and DU145 cells shows the regular signal pattern of abundant 28S and 18S rRNA. (B) RT-PCR experiments with gene-specific primers for the HSP89αΔN gene result in a reproducible product in LNCaP and DU145 cells, which does not map to the HSP89αΔN gene but to another HSP90 isoform on chromosome 3 (C). (D) Cloning strategy for HSP89αΔN with schematic overview of the primer positions used in the nested PCR approach.

Interestingly, my experimental analyses during the first four-month EWF resulted in the realization that the report of HSP89αΔN had been a cDNA synthesis artifact. At the time of proposal submission, two publications in international peer-reviewed journals had claimed the isolation of HSP89αΔN (Schweinfest et al., 1998; Grammatikakis et al., 2002). However, my highly sensitive nested RT-PCR experiments were unable to repoduce these results and my conclusion has been confirmed in an independent study by another research group, which was published in November 2008 (Zurawska et al., 2008). Consequently, it is not surprising that I was unable to prove the existence of the HSP89αΔN mRNA. Since Aim 2 of the original proposal rested entirely on the demonstration of HSP89αΔN as an HSP90 isoform involved in the lipid raft-recruitment of Akt (Figure 5 and 6 in the original proposal), it was necessary to develop an alternative hypothesis testing Akt recruitment.

During the second four-month EWF, I decided to embark on a different stategy to elucidate recruitment of the Akt1 kinase to lipid rafts in prostate cancer cells. As mentioned above, our laboratory has begun to specifically explore the lipid raft microdomain in search for Akt1 interacting proteins that transit to raft membranes. Based on this systematic search, we identified the heterogeneous nuclear ribonucleoprotein K (hnRNPK) as a Akt1 binding protein in lipid rafts. hnRNPK acts as a scaffold protein that integrates signaling cascades by facilitating the cross talk between kinases (Mukhopadhyay et al.,

2009). Therefore, I hypothesized that, similar to HSP90, hnRNPK could serve as a scaffold protein for Akt1 and may facilitate recruitment of Akt1 to the lipid raft compartment. To identify, which domain of hnRNPK is responsible for complex formation with Akt1 (an experiment that was originally proposed for HSP90 in Task 4. iii), I generated various truncated versions of hnRNPK and tested them in *in-vitro* GST binding studies (Fig. 10). The hnRNPK protein binds with high affinity to Akt1 as demonstrated by purification of GST-hnRNPK fusion proteins with subsequent western-blotting and detection of Akt1 with an Akt1-specific 2H10 antibody. Interestingly, deletion of the KH1 domain facilitates binding of Akt1 to hnRNPK, whereas the C-terminus seems to be important for Akt1 binding. These studies provided important clues to understand, which domains of hnRNPK are important for complex formation with Akt1 and whether hnRNPK can function as a scaffold protein that is instrumental in Akt1 kinase membrane recruitment.

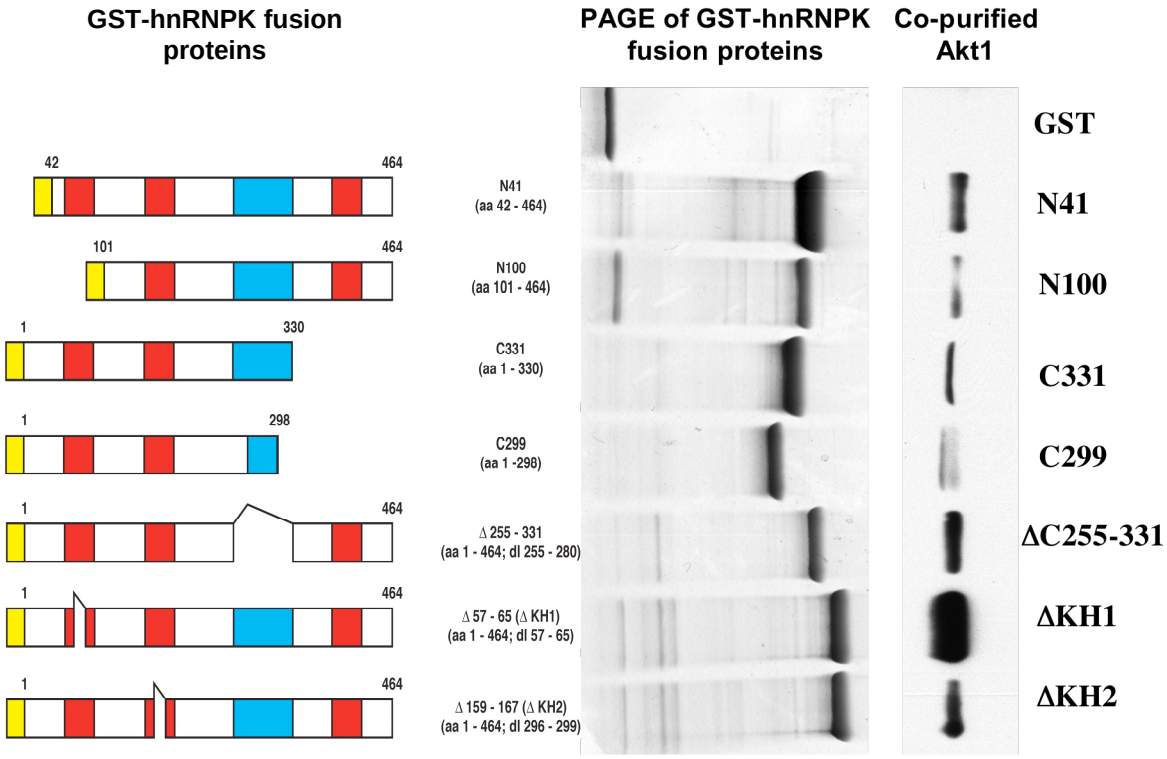

Figure 10: Domain structure of hnRNPK and complex formation with Akt1. An overview of the hnRNPK truncation mutants used in the binding assay as GST fusion proteins (left). Purification of GST-hnRNPK fusion proteins by affinity chromatography reveals co-purification of Akt1 (right).

To further elucidate whether it was possible to detect complex formation of Akt1 with hnRNPK in cholesterol-rich membrane rafts (an experiment originally proposed for HSP90 in Task 3), I isolated raft fractions using our established SDEM protocol to study subcellular localization. I was able to demonstrate that complex formation of hnRNPK with Akt1 was detectable in the raft compartment and exclusively confined to this membrane domain (Fig. 11). Consequently, I identified hnRNPK as a scaffold protein for the Akt1 kinase in the lipid raft compartment, which integrates signaling cascades by facilitating the cross talk between kinases and facilitating recruitment of Akt1 to the lipid raft compartment.

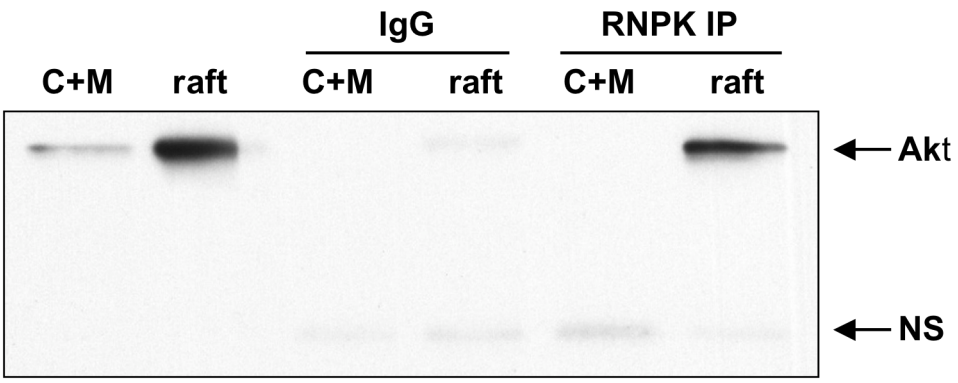

Figure 11: Association of hnRNPK and Akt1 in cholesterol-rich membrane microdomains. Immunoprecipitated hnRNPK from cytosol/non-raft membranes (C+M) or raft fractions (raft) displays complex formation with Akt1 only in the lipid raft domain.

KEY RESEARCH ACCOMPLISHMENTS

- Raft-resident Akt1 was identified as an important signaling molecule that confers a cytoprotective effect.
- Akt1 activity was shown to be specifically regulated in the raft compartment, which facilitates interaction of complex formation of Akt1 and its inhibitory kinase Mst1
- Myristoylated Akt1, which is an oncogene, is over-represented in lipid raft fractions in comparison to wild type Akt1 and mutations at important regulatory sites of Akt1 alter its lipid raft distribution.
- hnRNPK is a scaffold protein that is a key interaction partner for Akt1 in cholesterol-rich lipid rafts and facilitates recruitment specifically to this membrane compartment.
- This is the first evidence that cholesterol is a direct regulator of Akt-dependent signaling in prostate cancer cells.

CONCLUSION

In summary, I have obtained evidence that signals emanating from raft-resident Akt1 provide important cell survival cues. These signals are sensitive to cholesterol depletion suggesting that anti-apoptotic signals derive from Akt1 when localized to rafts. Consequently, the findings of my research as a Department of Defense Prostate Cancer Research Program Scholar suggest a direct mechanistic link between cholesterol and cell survival signaling in tumor cells and may be functionally relevant to the reported chemopreventive benefit of long-term use of cholesterol-lowering drugs in certain cancers.

REPORTABLE OUTCOMES

To date, publications attributable in whole or part to funding from this grant have been submitted or published:

- Nishit Mukhopadhyay, Jayoung Kim, Bekir Cinar, Aruna Ramachandran, **Martin H. Hager**, Rosalyn M. Adam, Pradip Raychaudhuri, Arrigo DeBenedetti and Michael R. Freeman (2009) Heterogeneous Nuclear Ribonucleoprotein K is a Novel Regulator of Androgen Receptor Translation. *Cancer Res.* 69: (6): 2210-2218.

This publication resulted from my studies on hnRNPK (Fig. 10, 11), which demonstrated that hnRNPK interacts with the Akt kinase but also with the androgen receptor in prostate cancer cells. The award W81XWH-07-1-0148 is mentioned in the acknowledgement section.

- Dolores Di Vizio, Jayoung Kim, **Martin H. Hager**, Matteo Morello, Marc A. Rubin, Rosalyn M. Adam, Wei Yang and Michael R. Freeman (2009) Oncosome Formation in prostate cancer: Association with a region of frequent chromosomal deletion in metastatic disease. *Cancer Res.* 69: (13) 5601- 5609.

This publication made use of my qRT-PCR expertise that I had gained during my studies on HSP89$\alpha\Delta$N. The award W81XWH-07-1-0148 is explicitly mentioned in the acknowledgement section.

- Nishit Mukhopadhyay, **Martin H. Hager**, Jayoung Kim, Delia Lopez, Dolores Di Vizio, Bekir Cinar, Rosalyn Adam and Michael R. Freeman (2010) The Akt1 and Mst1 signaling pathways converge on the steroid receptor co-repressor SAFB-1. (submitted)

This publication made use of my mRNA expression studies in various cancer cells, which I initiated in the course of this training award. The award W81XWH-07-1-0148 is explicitly mentioned in the acknowledgement section.

- **Martin H. Hager**, Ilona N. Holcomb, Wennuan Liu, Shizen Gao, Dolores Di Vizio, Francesca Demichelis, William N. Isaacs, Henry N. Higgs, Robert L. Vessella, Joan S. Brugge and Michael R. Freeman (2010) *DIAPH3* encodes a novel metastasis suppressor near the *RB1* locus. (submitted)

This publication resulted from studies on a side project during my time as a Department of Defense Prostate Cancer Research Program scholar. The award W81XWH-07-1-0148 is explicitly mentioned in the acknowledgement section. The abstract was selected for an oral presentation at the 2011 IMPaCT conference.

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